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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

PENN-0583

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/297877

INTERNATIONAL APPLICATION NO.

PCT/US97/20212

INTERNATIONAL FILING DATE

13 November 1997

PRIORITY DATE CLAIMED

15 November 1996

TITLE OF INVENTION

Screening for Modulators of Amyloid Processing

APPLICANT(S) FOR DO/EO/US

LEE, Virginia M.-Y. and DOMS, Robert W.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Executed Verified Statement Claiming Small Entity Status

"EXPRESS MAIL" Mailing Label No. 729215100009
Date of Deposit **10 May 1999**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20231

Suzanne Sparkman
(MAILER)
Suzanne Sparkman
(SIGNATURE)

U.S. APPLICATION NO. **09/297877**

INTERNATIONAL APPLICATION NO.

PCT/US97/20212

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PENN-0583

20. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

CALCULATIONS PTO USE ONLY

- ☐ Search Report has been prepared by the EPO or JPO \$840.00
- ☒ International preliminary examination fee paid to USPTO (37 CFR 1.482) \$670.00
- ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$760.00
- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$970.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$670.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	3 - 20 =	0	x \$18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$78.00	\$0.00	
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>	\$0.00	

TOTAL OF ABOVE CALCULATIONS =

\$670.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).

☒

\$335.00

SUBTOTAL =

\$335.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00

TOTAL NATIONAL FEE =

\$335.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐

\$0.00

TOTAL FEES ENCLOSED =

\$335.00

Amount to be:
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- ☒ A check in the amount of **\$335.00** to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **12-1086** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

LICATA, Jane Massey
Law Offices of Jane Massey Licata
66 E. Main Street
Marlton, New Jersey 08053 US
609-810-1515

SIGNATURE

Jane Massey Licata

NAME

32,257

REGISTRATION NUMBER

10 May 1999

DATE

Attorney Docket No.: PENN-0563
Inventor or Patentee: Virginia M.-Y. Lee and Robert W. Doms
Serial or Patent No.: PCT/US97/20212
Date Filed or Issued: November 13, 1997
Title: Screening for Modulators of Amyloid Processing

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION The Trustees of the University of Pennsylvania
ADDRESS OF ORGANIZATION 3700 Market Street, Suite 300
Philadelphia, Pennsylvania 19104

TYPE OF ORGANIZATION:

- ☒ (XX) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ () TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
- ☐ () NONPROFIT SCIENTIFIC OR EDUCATIONAL ORGANIZATION QUALIFIED UNDER A NONPROFIT ORGANIZATION STATUTE OF A STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
- ☐ () WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ () WOULD QUALIFY AS A NONPROFIT SCIENTIFIC OR EDUCATIONAL ORGANIZATION QUALIFIED UNDER A NONPROFIT ORGANIZATION STATUTE OF A STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **Screening for Modulators of Amyloid Processing** by inventor(s) **Lee and Doms** described in

- () the specification filed herewith.
(XX) application serial no. PCT/US97/20212, filed November 13, 1997.
() patent no. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities.
(37 CFR 1.27)

FULL NAME:

ADDRESS:

() **INDIVIDUAL** () **SMALL BUSINESS CONCERN** () **NONPROFIT ORGANIZATION**

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING

TITLE IN ORGANIZATION

ADDRESS OF PERSON SIGNING

Evalyn McConathy
Director, Intellectual Property
Center for Technology Transfer
3700 Market Street, Suite 300
Philadelphia, Pennsylvania 19104

Evalyn H. McConathy
SIGNATURE

May 5, 1999
DATE

SCREENING FOR MODULATORS OF AMYLOID PROCESSINGIntroduction

5 This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

Field of the Invention

10 Patients suffering from Alzheimer's disease are afflicted with neuritic plaques and vascular deposits consisting of amyloid fibrils made up of amyloid β peptides. Novel β -secretase enzymatic pathways which process amyloid β precursor peptide to amyloid β peptides have now been identified and are located in the endoplasmic reticulum. The
15 present invention relates to methods of identifying agents which increase or decrease processing of amyloid precursor protein into amyloid β peptides by contacting NT2N cells with the agent and measuring levels of amyloid β peptides formed in the endoplasmic reticulum of the cells. Agents identified to
20 increase processing of amyloid precursor protein into amyloid β peptides can be used in the early diagnosis of Alzheimer's disease while agents identified to decrease this processing are expected to be useful in the treatment of Alzheimer's disease.

Background of the Invention

25 Amyloid β ($A\beta$) peptides are the building blocks of the amyloid fibrils found in neuritic plaques and vascular deposits

- that accumulate in the brains of patients with Alzheimer's disease (AD; Selkoe, D. J. 1994 *Ann. Rev. Cell. Biol.* 10:373-403). A β is derived from proteolytic processing of one or more isoforms of the amyloid precursor protein (APP; Kang et al. 1987 *Nature* 325:733-736). APP isoforms are alternatively spliced type I transmembrane glycoproteins that are encoded by a single gene on human chromosome 21 (Kang et al. 1987 *Nature* 325:733-736; St. George-Hyslop et al. 1987 *Science* 235:885-890). The 39-43 amino acid long A β sequence begins in the ectodomain of APP and extends into the transmembrane region. Of the 3 major A β -containing isoforms encoded by the APP gene (i.e., APP695, APP751, and APP770; Kang et al. 1987 *Nature* 325:733-736; Ponte et al. 1988 *Nature* 331:525-527; Tanzi et al. 1988 *Nature* 331:528-530; Kitaguchi et al. 1988 *Nature* 331:530-532), APP695 is expressed almost exclusively by neurons of the central and peripheral nervous systems (Golde et al. 1990 *Neuron* 4:253-267; Kang, J. and Müller-Hill, B. 1990 *Biochem. Biophys. Res. Commun.* 166:1192-1200; Arai et al. 1991 *Ann. Neurol.* 30:686-693).
- Newly synthesized APP matures in the endoplasmic reticulum and the Golgi apparatus acquiring N- and O-linked carbohydrates, tyrosine sulfates (Weidemann et al. 1997 *Nature Med.* 3:328-332; Oltersdorf et al. 1990 *J. Biol. Chem.* 265:4492-4497) and phosphates (Oltersdorf et al. 1990 *J. Biol. Chem.* 265:4492-4497; Suzuki et al. 1992 *Neurosci.* 48:755-761; and Knops et al. 1993 *Biochem Biophys. Res. Comm.* 197:380-385). Several pathways of APP metabolism have been described in cultured cells, and evidence suggests that the relative importance of each pathway depends on the cell type. For example, non-neuronal cells preferentially process APP by the α -secretase pathway which cleaves APP within the A β sequence, thereby precluding the formation of A β (Esch et al. 1990 *Science* 248:1122-1124; Sisodia et al. 1990 *Science* 248:492-495). The putative α -secretase enzyme(s) is active at or near the cell surface, causing the N-terminal fragment (APP_N) to be quickly secreted. In contrast, neuronal cells process a much larger portion of APP by the β -secretase pathway(s), which

generates intact A β by the combined activity of two enzyme classes. The β -secretase(s) cleaves APP at the amino terminus of the A β domain releasing a distinct N-terminal fragment (APP β). In addition, the γ -secretase(s) cleaves APP at
5 alternative sites of the carboxy terminus generating species of A β that are either 40 (A β_{40}) or 42 amino acids long (A β_{42} ; Seubert et al. 1993 *Nature* 361:260-263; Suzuki et al. 1994 *Science* 264:1336-1340; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970).

10 A β_{42} is believed to have a significant role in AD pathology. Studies have shown that A β_{42} is more prone to formation of insoluble aggregates. Jarrett et al. 1993 *Cell* 73:1055-1058. Further, mutations in APP which increase the relative proportion of A β_{42} have been linked to familial AD.
15 Suzuki et al. 1994 *Science* 264:1336-1340. In addition, A β_{42} has been shown to be preferentially deposited in amyloid plaques. Iwatsubo et al. 1994 *Neuron* 13:45-53.

In vitro studies indicate the existence of at least two β -secretase pathways. In the endosomal/lysosomal pathway,
20 APP targeted to the cell surface is endocytosed and delivered to endosomes and lysosomes where β - and γ -cleavages can occur (Golde et al. 1992 *Science* 255:728-730; Nordstedt et al. 1993 *J. Biol. Chem.* 268:608-612; Haass et al. 1992a *Nature* 357:500-503; Koo, E. H. and Squazzo, S. 1994 *J. Biol. Chem.* 269:17386-
25 17389; Lai et al. 1995 *J. Biol. Chem.* 270:3565-3573; Perez et al. 1996 *J. Biol. Chem.* 271:9100-9107). The alternative β -secretory pathway is believed to generate A β in Golgi-derived vesicles, most likely secretory vesicles, prior to secretion (Haass et al. 1995a *Nature Med.* 1:1291-1296; Higaki et al. 1995
30 *Neuron* 14:651-659; Perez et al. 1996 *J. Biol. Chem.* 271:9100-9107; Thinakaran et al. 1996b *J. Biol. Chem.* 271:9390-9397).

Both A β_{40} and A β_{42} have been shown to be produced intracellularly from endogenous wild-type APP695 by cultured postmitotic CNS neuronal cells (NT2N) that are induced to
35 differentiate from a human teratocarcinoma cell line (NT2) by treatment with retinoic acid (Pleasure et al. 1992 *J. Neurosci.* 12:1802-1815; Pleasure, S. J. and Lee, V. M.-Y. 1993 *J.*

Neurosci. Res. 35:585-602; Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). To date, the human derived NT2N neuron is the only cell line documented to generate intracellular $A\beta_{40}$ and $A\beta_{42}$ before their eventual release into the medium (Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). Because neurons are the cell type most adversely affected by AD, the NT2N neurons represent a unique system for the study of APP processing and $A\beta$ biogenesis. An essential first step in the analysis of such pathways is the identification of the proteolytic fragments that are the products of these cleavages.

It has now been found that γ -secretase acts in the Endoplasmic Reticulum (ER) to yield $A\beta_{42}$. The N-terminal fragment generated by β -cleavage (i.e., APP β) has also been found to be produced by β -secretase intracellularly in NT2N neurons prior to secretion. These protease activities were identified to occur in the Endoplasmic Reticulum (ER)/Intermediate Compartment (IC) of neuronal cells utilizing inhibition with Brefeldin A (BFA), incubation at 15°C, and expression of exogenous APP bearing the di-lysine ER-retrieval motif. Accordingly, the present invention relates to methods of identifying compounds targeted to the endoplasmic reticulum which increase or decrease processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease.

Summary of the Invention

Novel β -secretase pathways have now been identified that produce amyloid β peptides, $A\beta_{42}$ and APP β , in the endoplasmic reticulum/intermediate compartment. Discovery of these enzymatic pathways and their location within the endoplasmic reticulum can be used to design new therapeutic approaches or agents which reduce production of amyloid β peptides. An object of the present invention is to provide a method of identifying agents which modulate processing of amyloid precursor protein into amyloid β peptides found in

neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease. In one embodiment, the method of the present invention may be used to identify inhibitors of this process which may be useful in the treatment of Alzheimer's disease. Alternatively, the method of the present invention may be useful in identifying agents or genetic mutations which increase this process, thereby increasing the formation of amyloid β peptides and the possibility of developing Alzheimer's disease.

10 Detailed Description of the Invention

APP serves as a substrate for a variety of proteolytic processing pathways, only some of which result in the production of $A\beta$ (Selkoe, D. J. 1994 *Ann. Rev. Cell. Biol.* 10:373-403). However, $A\beta$ is the major component of senile plaques in the AD brain. Moreover, mutations in the APP gene associated with Familial Alzheimer's Disease (FAD) alter APP processing and $A\beta$ production *in vitro* (Citron et al. 1992 *Nature* 360:672-674; Cai et al. 1993 *Science* 259:514-516; Suzuki et al. 1994 *Science* 264:1336-1340). Thus, determination of the proteolytic events that lead to $A\beta$ production and identification of the proteases responsible for each step as well as the sites of their action are important in the development of treatments for Alzheimer's disease and in the identification of causes for this disease.

In the present invention, the NT2N system was used to study APP processing in neurons. NT2N cells have been reported produce intracellular $A\beta$ (Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). NT2N neurons express the isoform of APP expressed almost exclusively in the CNS (i.e., APP695) and generate detectable intracellular levels of both $A\beta_{40}$ and $A\beta_{42}$. Further, they constitutively produce and secrete $A\beta$. Using antibodies specific to APP β and other proteolytic fragments, a number of intracellular β -secretase activities which cleave on the amino terminus side of $A\beta$ have now been identified and characterized. Moreover, novel β -secretase activities that

occur in a pre-Golgi compartment have now been identified.

For example, β -secretase(s) cleaves APP at the amino terminus of the A β domain releasing a distinct N-terminal fragment (APP β). To determine if intracellular APP β could be recovered from NT2N cells, samples of cell lysate were immunoprecipitated with Karen (an antiserum raised to the N-terminal region of APP). The presence of APP β in the immunoprecipitate was determined by immunoblot analysis using 53 (a polyclonal antibody specific for the free carboxy-terminus of APP β). This polyclonal antibody detected a single band of approximately 95 kDa. The identity of this 95 kDa APP fragment to be APP β cleaved at the β -secretase site was confirmed by: 1) the inability of 369W, an antibody specific for the C-terminus of APP, to recognize this fragment; 2) the inability of 6E10, an antibody specific for the first 10 amino acid residues of A β , to detect this fragment; 3) the binding of Karen, an antibody that recognizes all APP species, to this fragment; 4) the fact that this intracellular APP fragment is about 11-12 kDa smaller than APP_{FL}; and 5) the detection of the same 95 kDa APP fragment using a different antibody specific for APP β (i.e., 192; Seubert et al. 1992 *Nature* 359:325-327). APP β was readily detected in the media of NT2N neurons and co-migrated with APP β recovered from the cell lysates thus indicating that APP β was secreted.

The detection of intracellular APP β and A β in NT2N neurons indicates that β -secretase pathway including β - and γ -secretase occur in an intracellular compartment. The absence of intracellular APP α , however, suggests that the majority or all of the α -secretase activity occurs at a different site. To further confirm that the β -secretase pathways, but not the α -secretase pathway, occur inside these cells, the cell lysate of NT2N neurons was examined for the products of these respective pathways: A β , which is generated by β - and γ -secretase cleavages; and p3, a product of α - and γ -secretase cleavages. In these experiments, cell lysates of metabolically labeled NT2N neurons were immunoprecipitated with monoclonal antibodies (mAb) that can distinguish between these peptides: 4G8

recognizes both A β and p3; Ban50, however, binds only to A β and not p3. Data from these experiments clearly demonstrates that A β , but not p3, is produced intracellularly. The p3 fragment was not detected in cell lysates even after prolonged exposure of the film. By contrast, both A β and p3 were readily recovered from the media.

A series of experiments were performed which confirm that APP β is derived from APP_{FL} within the cell prior to secretion. In a first set of experiments, APP β was recovered from NT2N cell lysates even after intact NT2N neurons were treated with trypsin. Cultures of NT2N neurons were treated with trypsin at 4°C. Under such conditions, cell surface-associated but not intracellular APP β should be proteolyzed. However, a similar amount of APP β was recovered from NT2N neurons regardless of trypsin treatment. By contrast, when the NT2N neurons were treated with trypsin and 0.1% Triton X-100, intracellular APP β was completely eliminated. Thus APP β recovered from the NT2N cell lysate is indeed produced in an intracellular compartment.

A second set of experiments confirmed the continuous presence of steady state levels of APP β in NT2N neurons, together with a delay in the detection of APP β in freshly replenished medium. In these experiments, NT2N neurons were washed with fresh medium and the amount of intracellular as well as secreted APP β and APP α were measured over an 8 hour period by immunoprecipitation of cell lysates and media with Karen followed by immunoblotting with either antibody 53 (for APP β) or 6E10 (for APP α). Secreted APP β was first detected in 3 to 5 hours, and its accumulation in the medium continued over the 8 hour incubation period. In contrast, APP α was detected in one hour, suggesting that APP α is produced at a faster rate than APP β . As seen with APP β , APP α accumulated in the conditioned media over time. A steady state level of APP β was recovered from NT2N cell lysates prepared from parallel cultures over a period of 8 hours this indicating that intracellular APP β is produced constitutively.

Pulse-chase experiments were also performed which

demonstrated that the turnover of intracellular APP β lags behind the turnover of newly synthesized APP_{FL}, thereby confirming that APP β is generated from APP_{FL} inside NT2N neurons before secretion. This pulse-chase paradigm permits a more rigorous study of the temporal relationship between intracellular and secreted APP β . In these experiments, NT2N cultures were pulsed with [³⁵S]methionine for one hour and then chased for different lengths of time. After one hour of chase time, full length APP (APP_{FL}) immunoprecipitated from the cell lysate began to decline while the intracellular level of APP β continued to increase until 4 hours, after which it also declined. This lag in maximum production of intracellular radiolabeled APP β indicates that APP β is produced intracellularly from APP_{FL} by β -secretase cleavage. Further, the one hour delay in the secretion of APP β into the medium as well as the accumulation of this fragment with increasing chase time supports a temporal relationship between APP β that is produced intracellularly and APP β that is secreted into the medium.

The detection of APP β in the cell lysate of NT2N neurons, together with the presence of A β ₄₀ and A β ₄₂ (Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970), establish that an intracellular β -secretase pathway(s) exists in these cells. At present, no other cell line has been reported to produce detectable levels of intracellular APP β from endogenous or over-expressed wild-type APP (Seubert et al. 1993 *Nature* 361:260-263; Haass et al. 1995a *Nature Med.* 1:1291-1296; Thinakaran et al. 1996b *J. Biol. Chem.* 271:9390-9397). Only human kidney 293 cells stably transfected with APP_{sw} cDNA yield the related APP β _{sw} fragment from the cell lysates (Haass et al. 1995a *Nature Med.* 1:1291-1296; Martin et al. 1995 *J. Biol. Chem.* 270:26727-26730). In these non-neuronal cells, however, treatment with BFA completely eliminates APP β _{sw} and A β production (Haass et al. 1995a *Nature Med.* 1:1291-1296; Martin et al. 1995 *J. Biol. Chem.* 270:26727-26730; Essalmani et al. 1996 *Biochem. Biophys. Res. Commun.* 218:89-96). In contrast, NT2N neurons continue to produce APP β and A β during treatment

with BFA, implying that the subcellular site(s) of the β -secretase pathway is cell-type specific. In these experiments, NT2N neurons were metabolically labeled with [35 S]methionine in the presence or absence of 20 μ g/ml Brefeldin A (BFA). BFA is a pharmacological agent that causes a redistribution of the Golgi into the ER. In the absence of BFA, APP_{FL}, APP β , and A β were recovered from the cell lysates while APP α , APP β , and A β were detected in the media of NT2N neurons. Surprisingly, in the presence of BFA, not only APP_{FL} but also APP β and A β continued to be recovered from NT2N cell lysates. The effectiveness of BFA was verified by the fact that the secretion of APP α , APP β , and A β into the medium was completely abolished in its presence.

Cell lysates and medium from the cells were then analyzed using an ELISA system which quantitatively distinguishes between A β_{40} and A β_{42} to ascertain whether alteration of A β levels by BFA was the result of altered levels of A β_{40} , A β_{42} or both. Suzuki et al. 1994 *Science* 264:1336-1340. Results from the ELISA correlated with the immunoprecipitation data in that BFA abolished secretion of A β into the medium and reduced overall expression of intracellular A β by approximately 60%. However, it was the complete loss of A β_{40} that accounted for this decrease. A β_{42} was largely unaffected by BFA treatment. Thus, γ -secretases appear to be active in the endoplasmic reticulum, but only to yield A β_{42} . Further, it is believed that A β_{42} is preferentially generated in the ER while A β_{40} is generated in more distal components of the exocytic pathway.

Since BFA treatment results in retention of all newly synthesized proteins in the ER, additional experiments were conducted to confirm that the generation of A β_{42} in the ER was not due to retention of newly synthesized proteases not being delivered to their proper site of action. Accordingly, an ER-retention signal was placed in APP695 in which the third and fourth amino acids from the C-terminus were changed to lysine (APP695_{ΔKK}). This lysine motif is sufficient to retain heterologous transmembrane proteins in the ER and intermediate

compartment. In these experiments, recombinant Semliki Forest virus (SFV) vectors to express APP695_{AKK}. Previous studies have shown that despite high levels of SFV-mediated APP expression, SFV infected NT2N cells display a high degree of fidelity in processing APP (Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). Furthermore, it was found that cytopathic effects of SFV infection in NT2N cells as measured by LDH release do not develop until more than 48 hours after infection. To determine if APP695_{AKK} was in fact retained in the ER, indirect immunofluorescence microscopy was used to monitor both intracellular and cell surface distribution of SFV-expressed APP695 and APP695_{AKK}. While intracellular levels of APP695 and APP695_{AKK} appeared similar, only APP695 was expressed on the cell surface. BFA treatment blocked surface expression of APP695. In addition, APP695_{AKK} did not acquire resistance to endoglycosidase H digestion, a further indication that APP695_{AKK} is retained in the ER.

By retaining APP695_{AKK} in the ER, it was then determined whether A β is generated in this compartment without concomitant BFA-induced accumulation of other cellular proteins. SFV-infected NT2N cells were metabolically labeled overnight, and A β immunoprecipitated from the medium and cell lysate. It was found that ER retention of APP by the KK retention signal efficiently blocked A β secretion, yet failed to block all intracellular A β biosynthesis. Western blot analysis of intracellular and secreted APP showed that APP695 and APP695_{AKK} were expressed to comparable levels. As with BFA treatment, cells expressing APP695_{AKK} produced 40% of the total intracellular A β generated from APP695. Quantitative ELISA showed that this reduction was accounted for by the loss of A β ₄₀. A β ₄₂ levels were not affected. Thus, A β ₄₂ is cleaved from APP while still in the ER.

APP β recovered from BFA treated cells was found to migrate with an accelerated electrophoretic mobility compared to APP β from non-treated cells, suggesting that this fragment may have been derived from immature APP. To confirm that

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incomplete maturation of APP was the cause of the shift in electrophoretic mobility of the APP β fragment generated in the presence of BFA, NT2N cells were metabolically labeled with [³⁵S]methionine in the presence or absence of BFA. APP β immunoprecipitated from the cell lysate was then incubated with N-glycosidase F (Nglyc F), an enzyme that removes N-linked carbohydrate chains. APP β from BFA-treated NT2N neurons migrated more quickly than APP β recovered from untreated cells. After digestion with Nglyc F, APP β demonstrated a mobility downshift in SDS-PAGE. However, APP β from BFA treated cells still migrated faster than APP β from non-treated cells despite enzymatic removal of all N-linked carbohydrate chains. Thus, the increased electrophoretic mobility of APP β in the presence of BFA cannot be accounted for solely by differences in N-linked carbohydrate processing.

In addition to N-linked glycosylation, however, APP undergoes a variety of post-translational modifications, including the addition of O-linked carbohydrate chains. Therefore, both N- and O-linked carbohydrate chains were removed from immuno-precipitated APP β by simultaneous digestion with Nglyc F, O-glycosidase, and neuraminidase. Fully deglycosylated APP β co-migrated with APP β recovered from BFA treated NT2N neurons. Furthermore, combined BFA inhibition and deglycosylation did not induce a greater mobility shift than either of these treatments alone. Accordingly, APP β generated from BFA treated NT2N neurons is believed to represent β -secretase processing of immature (non-glycosylated) APP_{FL} in a pre-Golgi compartment.

To further verify that β -secretase cleavage occurs early in the biosynthetic pathway of NT2N neurons, an alternative non-pharmacological method to block protein transport from the ER to the Golgi was employed. Incubation of cultured cells at 15°C has been shown to inhibit newly synthesized proteins from exiting the intermediate compartment (Saraste, J. and Kuismanen, E. 1984 *Cell* 38:535-549; Saraste et al. 1986 *Proc. Natl. Acad. Sci. USA* 83:6425-6429; Schweizer et al. 1990 *Eur. J. Cell. Biol.* 53:185-196). In these

experiments, NT2N cells were incubated at 15°C for 16 hours. Only the immature form of APP_{FL} was present after a 16 hour incubation at 15°C as indicated by its sensitivity to Endoglycosidase H (Endo H) digestion, suggesting that it is not transported to the Golgi apparatus under these conditions. By contrast, incubation of the NT2N cells at 37°C yielded both immature and fully processed APP_{FL}. As expected, the immature APP_{FL} was Endo H sensitive, while the mature forms of APP_{FL}, having acquired post-translational modifications after exiting the ER, were Endo H resistant. In addition, secreted forms of APP were not detected in cells maintained at 15°C, further substantiating the effectiveness of the temperature block. Significantly, continuous production of intracellular APP β was observed at 15°C despite the fact that the secretion of APP ectodomain is completely abolished. Thus, these data also support the endoplasmic reticulum (ER)/intermediate compartment (IC) of NT2N neurons as a β -cleavage site.

The processing of wild-type APP695 and APP695 bearing the ER retrieval motif (APP695_{ΔKK}; Jackson et al. 1990 *EMBO J.* 9:3153-3162; Jackson et al. 1993 *J. Cell Biol.* 121:317-333) in the NT2N cells was also examined with regard to cleavage of APP β . To determine whether or not APP β can be produced from APP695_{ΔKK}, wild-type APP695 and APP695_{ΔKK} were separately expressed in NT2N neurons by infection with SFV vectors bearing these constructs. Following infection, duplicate wells containing wild-type APP695 infected cells were also treated with 20 μ g/ml BFA. The [³⁵S]methionine labeled cell lysates and the media were then sequentially immunoprecipitated with the antibodies 53 and Karen. Only the immature form of APP_{FL} was detected from cells expressing APP695_{ΔKK}. Significantly, intracellular production and secretion of APP β was not affected by genetic targeting of APP to the ER. Furthermore, it was found that unlike inhibition with BFA that eliminates transport of all proteins from the ER to the Golgi, specific retrieval of full-length APP695_{ΔKK} to the ER allowed the APP β fragment generated in the ER/IC to be transported to the Golgi complex for modification before secretion. Thus, it is believed that

once the ER retention motif is cleaved from the APP β fragment, it can then be transported to the Golgi complex for further maturation and subsequent secretion.

Generation of A β in the ER of NT2N neurons identifies these cells as a unique system in which to study amyloidogenic processing of APP and its role in the pathogenesis of AD. Mutations in both the APP gene and the recently identified presenilin genes are believed to cause AD by altering APP processing in ways that lead to the production of more amyloidogenic forms of A β (i.e., A β_{42} ; Scheuner et al. 1996 *Nature Med.* 2:864-870). Recently, in both non-neuronal and neuronal cells (including the NT2N neurons used in this study), the presenilin proteins have been localized to the ER (Cook et al. 1996 *Proc. Natl. Acad. Sci. USA* 93:9223-9228; Kovacs et al. 1996 *Nature Med.* 2:224-229; Thinakaran et al. 1996a. *Neuron* 17:181-190). Thus, the identification of amyloidogenic processing that occurs within the ER of neurons suggests that direct or indirect interaction may occur between the presenilins and APP. Furthermore, the mutations in the presenilin genes may alter this interaction in a manner that leads to increased production of APP β and ultimately A β_{42} . Accordingly, the identification of these secretase pathways in the ER of neuronal cells will permit the examination of the effects of both FAD-linked mutations occurring in the APP as well as the presenilin genes on the processing of APP in the ER. Further agents which modulate APP processing by increasing or decreasing production of APP β and A β_{42} can be identified by determining their effects on levels of APP β and A β_{42} produced by β - and γ -secretases in the ER of neuronal cells such as NT2N cells. Levels of peptides produced by this pathway can be routinely determined in accordance with teachings provided herein. Agents identified by this method to inhibit levels of APP β and/or A β_{42} produced by these enzymatic pathways may be useful in treating Alzheimer's disease while agents which increase levels of APP β produced by this pathway may be causative factors in the development of Alzheimer's disease.

The following nonlimiting examples are provided to

further illustrate the present invention.

EXAMPLES

Example 1 Cell Culture

NT2 cells derived from a human embryonal carcinoma cell line (Ntera 2/cl.D1) were grown and passage twice weekly in Opti-Mem (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) in accordance with procedures described by Pleasure et al. 1992 *J. Neurosci.* 12:1802-1815 and Pleasure, S. J. and Lee, V. M.-Y. 1993 *J. Neurosci. Res.* 35:585-602. To begin differentiation, 2.5×10^6 cells were seeded in a 75 cm² (T75) flask and fed with Dulbecco's modified Eagle's medium (DMEM) HG (Life Technologies, Inc., Gaithersburg, MD) containing 10 μ M retinoic acid, 10% FBS and P/S twice weekly for 5 weeks. The cells in a single T75 flask were then replated at a lower density in 2 x 225 cm² (T225) flasks for 10 days (Replate 1 cells). NT2N neurons with greater than 99% purity were then obtained by enzymatic treatment and mechanical dislodgment of Replate 1 cells and replated at a density of 6×10^6 cells per 10 cm dish previously coated with polylysine and Matrigel as described by Pleasure et al. 1992 *J. Neurosci.* 12:1802-1815. The NT2N neurons were maintained in medium consisting of one part conditioned medium and one part DMEM HG containing 10% FBS and P/S. For experiments involving the incubation of NT2N neurons at 15°C for 16 hours, regular medium containing DMEM HG and 10% FBS was replaced by DMEM HG containing 25 mM HEPES, 10% FBS, and P/S. Cultures of NT2N neurons were used for experiments when they were between 3 to 4 weeks old. CHO695 cells were grown and passed three times per week in α -MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS and P/S. M17 cells were grown and passage once per week in Opti-mem (Life Technologies, Inc., Gaithersburg, MD) containing 10% iron enriched calf serum and P/S.

**Example 2 Metabolic Labeling, Gel Electrophoresis,
Immunoblotting and Quantitation**

Cultured NT2N neurons were starved in methionine-free DMEM HG (Life Technologies, Inc., Gaithersburg, MD) for 30 minutes prior to incubation in fresh methionine-free DMEM HG containing 0.5 mCi/ml of [³⁵S]methionine (sp act. 1000 Ci/mmol; NEN-DuPont, Boston, MA). For steady-state labeling studies, NT2N neurons were labeled with [³⁵S]methionine continuously for 16 hours. For pulse-chase studies, cells were labeled with [³⁵S]methionine for 1 hour, washed twice with methionine-containing DMEM, and then chased in the same medium for 0 to 24 hours. APP_{FL}, APP α and APP β were separated on 7.5% Laemmli SDS-PAGE gels, and A β and p3 were separated on 10/16.5% step-gradient Tris-Tricine gels. These gels were either stained with Coomassie Brilliant Blue R (Pierce, Rockford, IL) and dried or transferred to nitrocellulose membranes and dried prior to exposure on PhosphorImager plates (Molecular Dynamics Inc., Sunnyvale, CA) for 3-5 days. The nitrocellulose replicas containing the immunoprecipitates were further probed with different antibodies in accordance with procedures described by Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517. Quantitation of bands in the autoradiogram was performed using the ImageQuant software (Molecular Dynamics Inc. Sunnyvale, CA) in accordance with procedures described by Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970. Radiolabeled proteins in SDS-PAGE gels and nitrocellulose replicas were also analyzed by standard autoradiographic methods. All experiments were repeated between 3 and 6 times.

**Example 3 Sample Preparation and Serial
Immunoprecipitations**

Cell lysates were prepared in accordance with procedures described by Golde et al. 1992 *Science* 255:728-730. Protein concentration was determined by the bicinchoninic acid procedure (Pierce, Rockford, IL). Media were centrifuged at 100,000 x g for one hour at 4°C before immunoprecipitation. Both cell lysates and media were precleared with protein A-

- Sephacrose (Pharmacia Biotech, Piscataway, NJ) in RIPA for one hour at 4°C. After recentrifugation at 15,000 x g for one minute, the supernatants were rocked overnight at 4°C with fresh protein A-Sepharose and the appropriate primary antibody.
- 5 After collecting the immunoprecipitates by recentrifugation at 15,000 x g for 1 minute, the supernatants were used in a second round of immunoprecipitation with fresh protein A-Sepharose and a different primary antibody.

Example 4 Trypsin Treatment of NT2N Neurons

- 10 NT2N neurons were metabolically labeled with 0.5 mCi/ml [³⁵S]methionine for 16 hours in accordance with the procedure set forth in Example 2. After rinsing the cultures twice with PBS, the NT2N neurons were incubated on ice for 20 minutes with PBS, with 10 µg/ml of trypsin in PBS alone (Life
- 15 Technologies, Inc., Gaithersburg, MD), or with 10 µg/ml trypsin and 0.1% Triton X-100 in PBS. Following this treatment, trypsin was inactivated by the addition of 100 µg/ml soybean trypsin inhibitor. The cells were then washed with PBS, scraped into cell lysis buffer, and processed for
- 20 immunoprecipitation as described in Example 3.

Example 5 Brefeldin A Treatment of NT2N Neurons and Deglycosylation of Immunoprecipitated APP β

- NT2N neurons were pretreated with 20 µg/ml of BFA for 1 hour before the addition of 0.5 mCi/ml of [³⁵S]methionine to
- 25 the cultures for 16 hours in the absence or presence of BFA. The cell lysates and media were processed for immunoprecipitation as described in Example 3. For deglycosylation of APP β , the immunoprecipitates containing APP β were washed twice in sodium phosphate buffer (20 mmol/l, pH
- 30 7.2) and boiled for 2 minutes in 10 µl of 1% SDS. The samples were then boiled for an additional 2 minutes after adding 90 µl of the sodium phosphate buffer with sodium azide (10 mmol/l), EDTA (50 mmol/l), and n-Octylglucoside (0.5% w/v). After this denaturation step, deglycosylation was initiated by the
- 35 addition of 2 mU Neuraminidase (Arthrobacter; Boehringer

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Mannheim, Indianapolis, IN), 2.5 mU O-Glycosidase (Boehringer Mannheim, Indianapolis, IN), and 0.4 U N-Glycosidase F (Boehringer Mannheim, Indianapolis, IN). The samples were then incubated at 37°C for 18 hours and deglycosylated APP β was run on 7.5% SDS-PAGE gels as described in Example 2.

For Endoglycosidase H (Endo H) sensitivity test, cell lysates and media were immunoprecipitated with Karen in accordance with procedures described in Example 3. Immunoprecipitates were recovered in 100 μ l 60 mM phosphate buffer, pH 5.7 with 1% SDS. The samples were then split in half (50 μ l each), and incubated with 4 μ l Endo H (Boehringer Mannheim, Indianapolis, IN) or vehicle at 37°C for 18 hours. The samples were then run on 7.5% SDS-PAGE gels in accordance with procedures described in Example 2.

15 **Example 6 Antibodies for Immunoprecipitation and Immunoblotting**

Karen is a goat polyclonal antisera raised to the large secreted N-terminal fragment of APP, and antibody 53 is a rabbit polyclonal antisera raised to a synthetic peptide corresponding to the amino acid sequence SEVKM. Antibody 53 binds specifically to the free C-terminus of APP β as disclosed by Howland et al. 1995 *Neurobiol. Aging* 16:685-699. Antibody 369W is a rabbit polyclonal antiserum raised to a synthetic peptide corresponding to the last 45 amino acid residues at the C-terminus of APP. Also used in this study were three monoclonal antibodies (MAb) to A β that are specific for residues 1-17 (6E10; Kim et al. 1988 *Neurosci. Res. Commun.* 2:121-130); residues 1-10 (Ban50; Suzuki et al. 1994 *Science* 264:1336-1340) and residues 18-25 (4G8; Kim et al. 1988 *Neurosci. Res. Commun.* 2:121-130).

Example 7 Preparation of SFV bearing pSFV-1(APP695) and pSFV-1(APP695_{AKK})

The di-lysine motif was introduced into APP695 by standard PCR site-directed mutagenesis of pSFV-1(APP695) using 35 primers 5'-CGAAAACACCGTGGAGCTCCTT-3' (SEQ ID NO: 1) and 5'-

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TTAACCCGGGCTAGTTCTGCTTCTTCTCAAAGAACTTGT-3' (SEQ ID NO: 2). The mutation containing PCR fragment was isolated by digestion with Bsm-1 and Xma-1, then ligated into pSFV(APP695) to yield pSFV(APP695_{ΔKK}). All pSFV-1 constructs, including a pSFV helper
5 plasmid with SFV structural genes, were linearized by digestion with Spe-1 and then used as a template for RNA synthesis with SP6 RNA polymerase. Co-electroporation of RNA from the expression and helper plasmids into BHK cells yielded infectious, replication-defective virus that was harvested 24
10 hours later in accordance with procedures described by Liljestrom, P. and Garoff, H. 1991 *Bio/Technology* 9:1356-1361. Accurate determination of viral stock titers were made in accordance with procedures described by Cook et al. 1996 *Proc. Natl. Acad. Sci. USA* 93:9223-9228. For all infection
15 experiments approximately 1×10^6 NT2N neurons per 35 mm dish were infected in serum free medium at a multiplicity of infection (MOI) of 7-10. When called for, 20 $\mu\text{g/ml}$ BFA was added after the completion of the infection step.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Virginia Lee, Robert Doms

(ii) TITLE OF INVENTION:

Methods of Identifying Modulators of Amyloid Precursor
Protein Processing in the Endoplasmic
Reticulum/Intermediate Compartment of NT2N Cells

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Jane Massey Licata, Esq.

(B) STREET: 66 E. Main Street

(C) CITY: Marlton

(D) STATE: NJ

(E) COUNTRY: USA

(F) ZIP: 08053

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM 486

(C) OPERATING SYSTEM: WINDOWS FOR WORKGROUPS

(D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:

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- 20 -

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Jane Massey Licata

(B) REGISTRATION NUMBER: 32,257

(C) REFERENCE/DOCKET NUMBER: PENN-0561

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (609) 779-2400

(B) TELEFAX: (609) 810-1454

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGAAAACCAC CGTGGAGCTC CTT

23

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTAACCCGGG CTAGTTCTGC TTCTTCTCAA AGAACTTGT

39

What is Claimed is:

1. A method of identifying agents which increase or decrease processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease comprising:

contacting NT2N cells with a compound or agent suspected of increasing or decreasing amyloid precursor protein processing; and

10 measuring levels of amyloid β peptides formed in the endoplasmic reticulum of the cells.

2. A method of diagnosing Alzheimer's disease in a patient comprising detecting in the patient an agent identified to 15 increase processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease in accordance with the method of claim 1.

3. A method of inhibiting processing of amyloid precursor 20 protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease comprising administering to the patient an agent which decreases processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and 25 vascular deposits that accumulate in brains of patients with Alzheimer's disease wherein said agent is identified in accordance with the method of claim 1.

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Screening for Modulators of Amyloid Processing**, the specification of which:

() is attached hereto.

(XX) was filed on 13 November 1997 as Application Serial No. PCT/US97/20212 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed			
			Yes		No	
			Yes		No	
			Yes		No	

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented)
60/030,967	Nov. 15, 1996	pending
60/055,299	Aug. 8, 1997	pending

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **Jane Massey Licata and Kathleen A. Tyrrell**, Registration Nos. **32,257 and 38,350**, respectively, of the firm of **Law Offices of Jane Massey Licata**, 66 E. Main Street, Marlton, New Jersey 08053, and

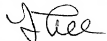

Address all telephone calls and correspondence to:

Jane Massey Licata, Esq.
Law Offices of Jane Massey Licata
66 E. Main Street
Marlton, New Jersey 08053

Telephone No.: (609) 810-1515

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1	Full Name: <u>1-00</u> <u>Virginia M.-Y. Lee</u>	Inventor's Signature: 	Date: <u>5/17/99</u>
	Residence: 2005 Pine Street Philadelphia, PA 19103 <u>PA</u>	Citizenship: United States	
	Post Office Address: Same as above.		
2	Full Name: <u>2-00</u> <u>Robert W. Doms</u>	Inventor's Signature: 	Date <u>5/19/99</u>
	Residence: 1230 Leopard Road Berwyn, PA 19312 <u>PA</u>	Citizenship: United States	
	Post Office Address: Same as above.		
3	Full Name:	Inventor's Signature:	Date
	Residence:	Citizenship:	
	Post Office Address: Same as above.		
4	Full Name:	Inventor's Signature:	Date
	Residence:	Citizenship:	
	Post Office Address: Same as above.		
5	Full Name:	Inventor's Signature:	Date
	Residence:	Citizenship:	
	Post Office Address: Same as above.		